A COMPARATIVE CYTOMORPHOMETRIC STUDY OF THE BUCCAL MUCOSA IN TOBACCO USERS (SMOKING, CHEWING AND COMBINED HABITS) AND NON-USERS

A Dissertation submitted
in partial fulfillment of the requirements
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BRANCH - IV

ORAL PATHOLOGY AND MICROBIOLOGY

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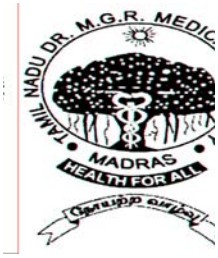

THE TAMILNADU DR. M.G.R. MEDICAL UNIVERSITY

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Certificate

THIS IS TO CERTIFY THAT **DR. L. BALASUBRAMANIAN**, POST GRADUATE STUDENT (2002-2005) IN THE DEPARTMENT OF ORAL PATHOLOGY AND MICROBIOLOGY, TAMIL NADU GOVT. DENTAL COLLEGE & HOSPITAL, CHENNAI-03 HAS DONE THIS DISSERTATION TITLED "***A COMPARATIVE CYTOMORPHOMETRIC STUDY OF THE BUCCAL MUCOSA IN TOBACCO USERS (SMOKING, CHEWING AND COMBINED HABITS)AND NON –USERS."*** UNDER OUR GUIDANCE AND SUPERVISION IN PARTIAL FULFILLMENT OF THE REGULATIONS LAID DOWN BY THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY, CHENNAI, FOR M.D.S., (BRANCH –IV ORAL PATHOLOGY AND MICROBIOLOGY) PART II DEGREE EXAMINATION.

DR. SHAHEEN AHAMED, M. D. S
PROFESSOR & H.O.D
Department of Oral Pathology & Microbiology
Tamil Nadu Government Dental College & Hospital, Chennai-3

DR. T.R. SUDARSAN, M.D.S.,
PRINCIPAL
Tamil Nadu Government Dental College
& Hospital, Chennai-3

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SL.NO	CONTENTS	PAGE NO.
1.	INTRODUCTION	1
2.	AIMS AND OBJECTIVES	4
3.	REVIEW OF LITERATURE	5
4.	MATERIALS AND METHODS	25
5.	RESULTS	31
6.	DISCUSSION	39
7.	SUMMARY AND CONCLUSION	44
8.	BIBLIOGRAPHY	47

INTRODUCTION

Cytology is the study of cells. Cellular morphology reflects the biologic behavior of the tissue and the host on one hand and the genetic and molecular biology of the cells themselves on the other. The science of cytopathology is based on these principles.

The rationale for exfoliative cytology lies in the epithelial physiology. Normal epithelium undergoes continual exfoliation of its superficial cells as a part of physiologic – turn over. The cells of the deeper layers are strongly adherent to each other under normal conditions. When the epithelium becomes the seat of malignant disease or of some benign conditions, the cells may lose their cohesiveness so that the deeper cells may be exfoliated along with the superficial cells.

The smear obtained by exfoliative cytology can be analyzed qualitatively as well as quantitatively. The parabasal squamous cells of the normal buccal mucosa have a rounded shape with well-defined cell border. The cytoplasm, which appears thick and homogeneous is stained blue / green with the PAP stain. The nucleus is darkly stained with a coarse chromatin pattern. The intermediate cells are polygonal in shape and while retaining an affinity for basophilic staining the cytoplasm taken on a more

transparent quality. There may be a yellow staining perinuclear region which denotes the presence of glycogen. The nucleus is round with fine chromatin structure. The superficial squamous cells are polygonal and have a pale pink –staining transparent cytoplasm with angular borders. The nuclei are small and structure less, being described as pyknotic. With advancements in the field of quantitative oral exfoliative cytology there has been a reemergence of oral exfoliative cytology as a powerful diagnostic tool. Various parameters such as nuclear size, cell size, nuclear cytoplamic ratio, nuclear shape, nuclear discontinuity, optical density and nuclear texture can be evaluated collectively in order to confirm the diagnosis accurately. Of these parameters, the nuclear and cytoplasmic areas and the nuclear to cytoplasmic ratios have been shown to be significant in the diagnosis of oral lesions. Because quantitative procedures are objective and reproducible, they may be important aids in making a cytopathologic diagnosis.

It has long been known that tobacco smoking play a major role in the etiology of oral cancer.

Tobacco was first used by the native Mexican Indians who inhaled the powdered leaves of the plant, the generic name of which is Nicotina. Tobacco was introduced in the Europe in the late 15th century. Sometime in the late 16th or early 17th century, Portugese

traders introduced it in India. Since then tobacco use has spread with remarkable rapidity seeping into all sections of the society. Initially tobacco was smoked in India, but later was used for chewing and application over the teeth and gingival as well (smokeless form). In India some 72% of tobacco users smoke bidis, 12% smoke cigarettes ad 16% use tobacco in the smokeless form.

Tobacco is addictive and is harmful to health in many ways. Cigarette, Bide, Cigar, Cheroot / Chuta , reverse Chutta smoking, Dhumti, reverse dhumti , chillum, hookah, are used for smoking tobacco. Smoke less tobacco include betel quid with tobacco, pan masala, mainpuri tobacco, mawa etc.

The government of India and the Stage governments have been taking efforts for primary prevention of tobacco use through various legislatures, mass medic etc. Oral exfoliative cytology can play a major role in the secondary prevention of tobacco habit by education and enlightening the tobacco user about his potential susceptibility to oral precancerous and cancerous lesions.

The present study has been undertaken to assess the cytomorphometric changes like cell diameter and nuclear diameter of the squames from clinically normal buccal mucosa of tobacco users (smokers, chewers and combined habit group) and non-users of tobacco and to compare and correlate the findings with age and sex.

AIMS AND OBJECTIVES

The aim of this study is,

to evaluate the cell diameter(CD) and nuclear diameter (ND) of squames from clinically normal buccal mucosa of

a. Non-users of tobacco.

b. Smokers.

c. Tobacco chewers.

d. Smokers and tobacco chewers.

To compare and correlate the findings based on the above groups with gender and age.

REVIEW OF LITERATURE

Exfoliative cytology:

The application of cytodiagnosis as a routine procedure in the early detection of cancer of the uterine cervix was introduced by George N. Papanicolaou in 1941. His work along with Traut conclusively demonstrated that cytodiagnosis could discover unsuspected uterine cancer and that the findings were reliable. Cytologic diagnosis has since been widely used not only in gynecological practice as “PAP test” but also in other sites of the body that elude ordinary diagnostic methods.

Ziskin et al in (1941)[67] first introduced the use of exfoliative cytology in the oral cavity. Miller and Montgomery in 1951 studied the normal exfoliative cytology of the oral mucosa and later Montgomery and Vohamm introduced it for the diagnosis of cancer.

Hannah Peters et al in (1956)[17]**,** suggested that exfoliative cytology is of immense use in studying lesions that are not to be biopsied ordinarily or in patients in whom surgical intervention is contra indicated.

Gustav et al (1963)[16] studied 40,373 oral smears and concluded that oral cytology represents a reliable and accurate technique in the diagnosis of benign and premalignent oral lesions.

They compared oral cytology and vaginal cytology and concluded that exfoliative cytology is more useful in early detection of cervical cancers as the period of carcinoma -in-situ is longer than oral cancers. Oral cancers are infiltrative at an earlier stage than vaginal cancers.

Ordie H. King, Hr. (1963)[45] describes the advantages of exfoliative cytology. Numerous innocent lesions often seen in examination of the oral structures may closely resemble early carcinoma. Common lesions such as herpetic ulcers, aspirin burns, secondarily infected traumatic lesions pigmented areas, smoker's stomatitis, hyper keratotic areas of various sizes as well as more uncommon ones such as primary and tertiary syphilitic lesions, tuberculous ulcerations and white sponge nevi are listed among these. If the dentist were to take a biopsy of all the commonly found lesions in the oral cavity, he would spend a large part of his time doing only this. Also there is considerable apprehension as well as discomfort on the part of the patient, associated with surgical wounds in the oral cavity. The ease, accuracy and lack of trauma offered by the use of the cytology technique make this an ideal method for the detection of early intra oral malignant neoplasm.

Joel W. Golds by et al (1964)[25] studied the nuclear and cell size of normal epithelial cells. Their study showed that the mean

nuclear size varied from 7.6μ to 10.9μ. The mean cellular diameter varied from 29.7μ to 98.21μ. The normal nuclear diameter is shown to be that of 1/6th of the cytoplasmic diameter.

Zimmermann. R.E., and Zimmermann A.L (1965)[66] studied the effects of Race, age, smoking habits and oral and systemic diseases on oral exfoliative cytology. The results indicated1) There was no statistically significant differences in mean cell count between races in any site. 2) Aging was associated with decreased keratinization in the gingival and hard palate 3) in all sites, a slightly increase in number of keratinized cells were found in smokers. 4) Patients with recent systemic disease had a significant reduction in keratinized cell count of the buccal mucosa compared to controls.

The **Editorial of JADA,** (1967)[13] has described the usefulness of exfoliative cytology in dentistry. The cytological technique has been shown to be an accurate and valuable aid not only for the early detection of uterine cancer but also for the early detection of oral cancer. It is not intended to support biopsy procedures but rather to be an aid to the general dental practioner in determining whether a clinically abnormal appearing area in the mouth is cytologically negative, suspicious or positive for malignant cells. The majority of very early malignancies cannot be characterized clinically and may be unnoticed or passed off as minor irritation that will heal in a few

weeks. Exfoliative cytology provides the dentist with a quick, painless and uncomplicated method of examining a slightly suspicious lesion.

Sigmund Stahl et al (1967)[52] stated that the accuracy of oral cytology screening for the diagnosis of oral cancer is high and "as high or even higher than that claimed for vaginal cytology".

Silverman S. Jr et al (1967)[54] in their study on the cytology of oral mucosa subsequent to radiation therapy have shown that there is an increase in nuclear and cytoplasmic areas, vacuolation, binucleation and nuclear aberration in patients who received radiation therapy.

Levin and Lunnin (1969)[30] in their study of exfoliative cytology in pemphigus vulgaris cases, identified acantholytic cells which have large single or multiple nuclei with increased nuclear cytoplasmic ratio.

William W. Johnston et al (1970)[65] studied the cytology of parotid duct secretions in various conditions. They found columnar epithelial cells in normal parotid secretions, malignant cells in adenocarcinoma, histiocytes and neutrophils in inflammatory conditions. They concluded that this procedure may greatly aid in diagnosing inflammatory and neoplastic conditions of salivary gland.

Reddy C.R. et al (1975)[48] in their study of oral exfoliative cytology in female reverse smokers having stomatitis nicotina have found that the karyopyknotic index increased in palatal and lingual mucosa when compared to non-smokers, The buccal mucosa did not show any change. There was also no change with age in the non- smoking females.

Hillman et al (1976)[20] evaluated papanicolaou smears from the cheeks and tongues of smokers and non-smokers for possible association between cytologic features and cigarette consumption. Their findings showed increase in nuclear size and decrease in cell size in smokers compared to normal. These findings appear consonant with the view that the microscopic changes are frequent in clinically unremarkable areas of the mouth and that tobacco induces some of the cellular changes to which its use has been clinically and epidemiologically related.

Masadomi et al (1978)[33] studied the effect of tobacco smoking and age change on the keratinization of the palatal mucosa using exfoliative cytology. They concluded that

a. There is greater degree of Keratinization in smokers than in non-smokers.

b. There is no statistically significant age difference in Keratinization.

Cowpe and longmore (1981)[7] studied the DNA content status in normal buccal smears and suggested that the mean nuclear area can be used as a baseline and can be compared with the abnormal cells.

Stitch HF et al (1984)[56]**,** studied the frequency of micro nuclei in the cells of the buccal mucosal smears. They concluded that the carcinogenic stimuli increase the frequency of micronuclei, which is a measure of chromosome breakage in cell divisions. They also found that the supplementation of diet with retinol and beta – carotene substantially decreased the mean proportion of cells with micronuclei in their study group of betel chewers where as the unsupplemented control group betel chewers did not show any change. This suggests the possibility that increase in dietary intake of retinol and / or carotene may reduce the incidence of oral cancer in betel chewers.

Cowpe (1984)[8] studied the cells of normal and malignant lesions using exfoliative cytology and found that there is increase in mean nuclear area, mean nuclear cytoplasmic ratio and significant reduction in cytoplasmic areas in malignant lesions.

Cowpe et al (1985)[9] described the development of quantitative cytological techniques and their application to oral smears. Nuclear and cell size have been measured and matched with age, sex and site in an attempt to produce a baseline for comparison with identical measurements carried out on pathological smears. The results displayed a significant variation in nuclear and cytoplasmic areas between different sites. Nuclear size varied significantly with advancing age. However this was not the case for cytoplasmic area. There was no significant variation in either criterion between males and females.

Cowpe J.G et al (1988)[10] quantitatively analyzed the smears collected from the buccal mucosa and the floor of the mouth. The results displayed an encouraging success rate for identifying premalignant and malignant lesions. Their results indicated that quantitative cytology could be of great value for monitoring and follow up of suspicious lesions and provide an excellent additional diagnostic test for detecting early oral malignancy.

Cytomorphometric study by **Van Der Wal N. et al** (1989)[63] in pseudo epitheliomatous hyperplasia of granular cell tumours, well differentiated squamous cell carcinoma and normal tongue for mean area, mean perimeter and the mean diameter of 50 largest squamous epithelial nuclei in 50 fields. The results were found to be

significantly larger in squamous cell carcinomas than in granular cell tumours and normal tongue.

Ogden and Cowpe (1989)[39] studied cytological smears of patients who received radiotherapy. They compared the values of nucleus and cytoplasmic areas in the smears obtained from buccal mucosa before and after radiotherapy. They concluded that the nuclear and the cytoplasmic areas increased after radiotherapy.

Ogden GR, et al (1990)[42] studied two groups, 40 patients with malignant disease outside the oral cavity and 40 healthy patients attending for routine dental treatment. Quantitative cytologic assessment of nuclear (NA) and cytoplasmic area (CA) were calculated from cells in normal buccal mucosal smears. No significant difference in NA (P=0.28) was found between the two groups. However a significant reduction in CA (P = 0.005) was found within the distant malignancy group. The conducted that the buccal smear may prove of value in assessing nutritional deficiency consequent to internal malignancy.

Ogden et al (1990)[43] assessed the effect of cigarette smoking on the oral mucosa through the application of quantitative cytomorphometric analysis to smears obtained from clinically normal buccal mucosa. The nuclear area (NA) and cytoplasmic area (CA) of cells within each smear were measured. Mean values for NA

(Smokers – 3273.9 microns^2, non smokers – 3098.96 microns^2) were obtained. These results suggest that for normal buccal mucosa, smoking appear to influence cytomorphology.

Ogden et al (1991)[40] quantitatively assessed the nuclear area and cytoplasmic area of smears taken from normal buccal mucosa of 76 cancer free control subjects and 55 oral cancer patients. A significant reduction in cytoplasmic area was observed in the smears of the oral cancer group, but no change in nuclear areas.

Jin. Y and Yang L.J. (1995)[23] analyzed the nuclear cytoplasmic ratio (N/c) and nuclear volume densities (V_{vn}) in defined strata from human hard palate lesions with and without malignant potential, to determine the diagnostic reliability and validity of these Parameters Measurements of cellular and nuclear areas of basal and spinous cells from normal and pathological palatal epithelium were made on histological sections using an image analyzer. The results indicated that N/c ratio seemed to be of no value as a predictor of malignancy in palatal epithelial lesions.

Joel – Epstein (1995)[24] studied smears from 30 patients with oral hairy leukoplakia by exfoliative cytology. PAP stained smears showed peripheral margination of nuclear chromatin. He concluded that exfoliative cytology is a simple, non-invasive procedure that can be used to confirm the clinical diagnosis of hairy leukoplakia.

Sugarman PB & Savage NW (1996)[57] reviewed the value of exfoliative cytology in oral cancer screening programmes as a public health measure and concluded that it is an excellent, rapid, non-invasive procedure for assessing dysplastic change within the oral epithelium.

Ramaesh et al (1998)[46] studied the quantitative parameters like nuclear diameter, cell diameter, and nuclear – cytoplasmic ratio in patients with leukoplakia and squamous cell carcinoma. They also selected 40 volunteers with normal mucosa as control group. They identified that there is a reduction in cell size from normal to dysplasia to carcinoma and suggested that it may be an early indication of malignant change. In contrast to cell size, the nuclear diameter increased in dysplasias and carcinomas.

Cowpe et al (1998)[11] in their quantitative study of squames collected from premalignat & malignant lesions revealed a sequential decrease in cell size through normal mucosal lesions with no dysplasias, dysplasia to squamous cell carcinoma. The authors suggested that this could be an early indication of malignant change. Intra patient normal smears provided a satisfactory control for comparison with pathologic smears.

Ramaesh et al (1999)[47] studied quantitatively the smears from the buccal mucosa of people having the habit of tobacco

chewing and smoking and non – users of tobacco and betel nut by exfoliative cytology. Nuclear diameter and cell diameter were measured. Nuclear diameter and cell diameter were found to increase and decrease respectively in tobacco users. They suggested that the cytomophometric changes could be produced by an inflammatory change due to chromic irritation of the oral mucosa caused by tobacco habits.

Ogden (1999)[42] studied the effect of alcohol on the clinically normal oral mucosa using quantitative cytomorphology. The results were compared to a control group of non-users of alcohol. The result showed a statistically significant reduction in mean cytoplasmic area and nuclear area for the alcohol group when compared to the control group.

Sampaio et al (1999)[50] compared the AgNOR count of the cells collected from the normal buccal mucosa of cigarette smokers with that obtained from non-smokers. The mean AGNOR count was statistically higher in cells of smokers than non-smokers. They concluded that cigarette smoking influences proliferative activity in the cells of normal buccal mucosa.

Bijoy Kumar Das (2000)[3] reviewed the non–invasive technique of exfoliative cytology and described it as the study of superficial cells which have been either exfoliated or shed naturally

from mucous membrane, renal tubules etc. Apart from using for the early diagnosis of cancer, it can be used in vesiculo bullous lesions, anemias and to identify sex chromosomes. It is of less use in non-ulcerative lesions. He also describes the merits of exfoliative cytology as a feasible method in oral epidemiological survey and as an adjuvant in diagnosing carcinomas. Authors also discuss the disadvantages like lesser information provided in the literature compared to histological preparation.

Vivek K. Pakmode (2000)[64] studied cytological smears from patients with oral pemphigus and showed the presence of acantholytic cells. The cells were present in isolation or in clusters, which were round in shape with serrated cytoplasmic membrane. The nucleoli were prominent and darkly stained. The author suggests the usage of this non-invasive technique for the early diagnosis of pemphigus.

Marcelo et al (2001)[31] studied patients with clinical suspicion of paracoccidioidomycosis. Smears were obtained and stained with methenamine silver for fungus. All the smears revealed the presence of P. brasiliensis in various shapes like round, banana, half moon, steering wheel etc. He concluded that exfoliative cytology can be used in the diagnosis of paracoccidioidomycosis.

Effect of tobacco on oral mucosa:

Jayant k et al (1977)[22] The etiologic fractions due to smoking and chewing tobacco have been quantified for the first time for cancers of the oral cavity, oropharynx, hypopharynx, larynx and oesophagus. The overall etiologic fractions due to smoking and or chewing tobacco have been found to be 70% for cancer of the oral cavity. Their study showed that the two factors, smoking and chewing acted synergistically though in varying degrees at different sites.

Bhonsle et al (1979)[2] The habit of chewing tobacco along with lime in the canine – premolar region of the mandibular groove is widespread (28%) in the rural population of Maharastra, India. Among individuals with this habit, a thick yellowish – white – to brown lesion was observed at the site of placement of the mixture. Unlike leukoplakia, this lesion could be scrapped off. They suggested that the mixture of tobacco – lime exerts a keratinizing and a caustic influence on the oral mucosa.

Mehta (1981)[34] In a prospective epidemiological house to house survey of a random sample in the district of Ernakulam in Kerala State. The annual incidence rate of leukoplakia per 1000 adults was found to be 2.1 for males and 1.5 for females. The rate

was highest in the mixed tobacco habits group and lowest in the no habit group.

Stich and Rosin (1983)[55] Studied quantitatively the synergistic effect of smoking and alcohol consumption on oral mucosa. Their result showed that there was an increase in the number of micronuleated buccal mucosal cells in smokers and alcoholics.

Binnie et al (1983)[4] in their report have discussed the etiological factors related to oral squamous cell carcinoma namely tobacco, alcohol, syphilis, oral sepsis, iron deficiency, chronic candidiasis and herpes simplex virus.

Hirsch J.M et al (1984)[18] in their study of effect of tobacco snuff and herpes simplex virus – 1 on rat oral mucosa found that the rats exposed to snuff alone or in combination with HSV –1 had a higher incidence of tumours or tumour like conditions than control rats exposed to HSV – 1 alone.

Chester W. Douglass et al (1984)[6] stated that sunlight and smoking were highly likely risk factors for the development of lip cancer. He stressed for further research into the their inter relationship and interaction with other factors such as genetic predisposition.

Oliver DE and Shillitoe E.J. (1984)[44] in their study of the effect of tobacco smoking, concluded that the carrier state of candida albicans is slightly increased in cigarette smokers.

Offenbacher S And Weathers D.R (1985)[38] in their study of possible relationship between smokeless tobacco usage and the presence of gingivitis, gingival recession, mucosal pathology and caries concluded that smokeless tobacco usage caused increased prevalance of gingival recession, mucosal pathology and caries experience in individuals with co-existing gingivitis.

Valentine J.A (1985)[60] in his study has shown that alcohol drinking or tobacco smoking is associated with structural changes in human lingual epithelium in the absence of clinical evidence of oral mucosal disease. The predominant change was one of atrophy manifested by a reduction of the mean epithelial thickness due to reduction in mean cell size.

Hirsch JM (1986)[19] made experimental study of the snuff-induced lesions in rats. His study revealed that the rats exposed to snuff for 13 months exhibited hyperplastic, hyper orthokeratotic epithelium with focal mild atypia, focal alteration and marked subepithelial fibrosis. These changes were markedly reduced or absent in rats exposed to snuff and killed after a snuff free interval of 1 or 4 months.

Chen S.Y. (1989)[5] in his study of the effects of smokeless tobacco on the buccal mucosa of HMT rats found hyper orthokeratosis, acanthosis, numerous binucleate spinous cells and subepithelial connective tissue hyalinization. 25% of buccal epithelial cells of the tobacco treated rats were tetraploid and 5% octaploid compared to only 11% tetraploid and no octaploid in the controls. The results indicate that the mitotic process can be disturbed by tobacco treatment.

Grady et. al (1990)[14] assessed the risk posed by smokeless tobacco on oral mucosa. Leukoplakia was very strongly associated with the use of smokeless tobacco. The amount of smokeless tobacco used (in hours per day that smokeless tobacco was held in the mouth) recency of its use (hours since last use), type (snuff versus chewing tobacco) and brand used were significantly associated with risk for leukoplakic lesions among smokeless tobacco users.

Nandakumar A. et al (1990)[35] The relative risk due to pan tobacco chewing was elevated in both males and females in a case-control study on the cancers of the oral cavity from the population based cancer registry in Bangalore. A statistically significant dose response based on years, times per day and period of time chewed were seen.

Gould M l. et al (1991)[15] oral cancers caused by chewing tobacco are common in India and some parts of the Indian subcontinent. The present study shows a significant association (P less than 0.001) between the use of Indian chewing tobacco and oral cancer. Number of quids, mean quantity of tobacco and the mean duration of keeping the quids in the mouth had direct dose and effect relationship in the causation of oral cancer.

Troy E.D. et al (1992)[59] studied the effect of the use of smokeless tobacco from the mucosal lesions in professional baseball players. Hyper parakeratosis, hyper orthokeratosis pale surface staining and basal cell hyperplasia were found in the tobacco users. Their study showed that the variety and severity of epithelial changes were more in snuff users than in tobacco chewers.

Andreas Joranovic et al (1993)[1] evaluated the possible relationship between tobacco smoking and alcohol consumption and the site of occurance of squamous cell carcinoma of the oral cavity. He found that the squamous cell carcinoma of the floor of the mouth and retromolar area were significantly more related to the use of tobacco and alcohol than squamous cell carcinoma of the tongue and cheek mucosa.

Jawdet D. Al-Damouk (1993)[21] stated that chronic alcohol ingestion caused significant atrophy of the posterior lateral tongue

epithelium which might render that area more susceptible to carcinogenesis.

Valerie A. Murrah et al (1993)[61] in his in-vitro studies, had directly assessed the potential of smokeless tobacco as oral carcinogen. Their results appeared to support their in vivo findings that the tobacco specific N–nitro amines were related to epithelial transformation in the oral cavity.

Day G.L. et al (1994)[12] Tobacco smoking and alcohol drinking each contributed to risk of second primary cancer with the effects of smoking more pronounced than those of alcohol.

Summers R. M et al (1994)[58] The high prevalence of oral cancer in South Asia has been linked to tobacco use particularly in conjunction with chewing betel quid or 'pan'. The use of tobacco and 'pan' imply an increased risk of oral cancer and pre cancer.

Ko Y.C et al (1995)[28] studied (a) relationship between the use of betel quid chewing, cigarette smoking, alcohol drinking and oral cancer (b) synergism between these factors. They concluded that the incidence of oral cancer was 123 fold higher in patients who smoked, drank alcohol and chewed betel quid than in abstainers. Swallowing of betel quid juice (Saliva extract of betel quid produced

by chewing) or including unripened betel fruit in the quid both seemed to enhance the risk of contracting oral cancer.

Kulasegaram R. et al (1995)[29] Their study reaffirms the role of dental practioners in identifying individuals at risk of oral mucosal disease, the importance of public education about the risk factors and the necessity for counseling patients with precancerous lesions and on avoiding further risk due to tobacco habits.

Reichart PA et al (1996)[49] have examined the effect of betal chewing on elderly Cambodian women. They have found out that 60.8% of the patients had a tendency to desquamation or peeling of the oral epithelium and 2.9% had leukoplakia.

Johnson G.K. et al (1998)[29] The most prevalent oral manifestation of smokeless tobacco use is a white mucosal lesion at the habitual site of tobacco placement. The lesions develop due to the chemical or physical irritating properties of the tobacco quid. Tobacco exposure stimulates production of inflammatory mediators and cytokines such as prostaglandin E2 (PGE2) and interleukin –1 (IL-1) both of which participate in epidermal inflammation and epithelial proliferation.

F.A. Khan et al (2000)[27] The high incidence of oral cancer in some population groups is thought to be related to the cultural habits

such as chewing pan and tobacco in South Asia and the greater use of alcohol in parts of Western Europe.

Vander Biji et al (2001)[62] Arecanut is used raw, dried, baked or boiled and pickled. The adverse effects associated with arecanut chewing include oral cancer and oral sub mucous fibrosis because of their genotoxic, mutagenic and carcinogenic potential which are due to arecoline and arecaidine. The accumulation of these cytotoxic compounds from arecanut in the epithelial layer might result in irritation and inflammation as well as impaired collagen turn over with irreversible fibrosis and carcinogenesis.

Marina et al (2002)[32] The tobacco snuff does not cause epithelial thickening by inducing hyperproliferation of epithelial cells, but rather by extending the life span of differentiated cells before desquamation.

MATERIALS AND METHOD

Sample Collection:

80 patients who visited the department of Oral Medicine and Radiology. Tamilnadu Government Dental College and Hospital, Chennai were screened using mouth mirror and probe with adequate illumination and smears were taken from buccal mucosa.

Selection Criteria:

Patients, who have the habit of tobacco smoking, chewing, combined habits and control group without any of these habits were included in the survey. Patients with known history of chronic infections like tuberculosis, AIDS, bleeding disorders, anemias and other systemic disorders were excluded. Patients with oral mucosal lesions were not selected.

Materials used: (for sample collection and smear preparation)

1. Mouth mirror
2. Gauze
3. Pair of tweezers
4. Wooden spatula
5. Glass slides
6. 95% ethyl alcohol
7. Glass marking pencil

The subjects were asked to rinse their mouth with clean water. Following this, scrapings were made from the buccal mucosa of the subjects with a wooden spatula. The scrapings were smeared on the glass slides over an area of about 2.5x2 cm. The slides were fixed by placing in 95% ethyl alcohol immediately without allowing the slides to dry. The slides were fixed for a minimum of one hour.

STAINING TECHNIQUE:

The universal stain for cytological preparations is the papanicolaou stain. Harris haematoxylin is the optimum nuclear stain and the combination of OG 6 and EA 50 give the subtle range of green, blue and pink hues to the cell cytoplasm.

Materials used:

1. Distilled water
2. Papanicolauo staining kit which contains
    - Harris Haematoxylin
    - Orange II
    - EA-50
3. HydroChoric acid
4. Xylene

Papanicolaou staining:

The fixed smears were stained using Papanicolaou staining method as follows:

1.	Hydrate in 95% alcohol	-	2 minutes
2.	Hydrate in 70% alcohol	-	2 minutes
3.	Rinse in Water	-	1 minutes
4.	Stain in Harris Haematoxylin	-	5 minutes
5.	Rinse in water	-	2 minutes
6.	Differentiate in 0.5% aqueous HCL	-	10 seconds
7.	Blue in 1% Ammoniated water	-	2 minutes
8.	Rinse in water	-	2 minute
9.	Dehydrate in 70% alcohol	-	2 minutes
10.	Dehydrate in 95% alcohol	-	2 minutes
11.	Dehydrate in 95% alcohol	-	2 minutes
12.	Stain in Orange – II	-	2 minutes
13.	Rinse in 95% alcohol	-	2 minutes
14.	Rinse in 95% alcohol	-	2 minutes
15.	Stain in EA – 50	-	3 minutes
16.	Rinse in 95% alcohol	-	1 minutes

Interpretation:

- Nuclear appears blue / black
- Cytoplasm of non keratinising squamous cells appear blue/ green
- Cytoplasm of keratinising cells appear pink / orange

CYTOMORPHOMETRIC ANALYSIS

Materials used:

1. Compound microscope
2. Eyepiece micrometer
3. Stage micrometer
4. Immersion oil

Standardisation of micrometer:

1. The eye piece micrometer scale is fixed inside the eye piece lens of the microscope.
2. The stage micrometer is placed on the stage of the microscope.
3. The stage micrometer is focused with 100x objective in oil immersion.
4. The number of divisions of the eye piece micrometer that coincides with any of the stage micrometer is found out.

From this the actual size of each division of eye piece micrometer can be calculated as follows:

1 division of stage micrometer = 0.01mm i.e. 10μ

11 divisions of eye piece micrometer coincides with 1 division of stage micrometer which is equal to 10μ.Therefore 1 division of eye piece micrometer equals 10/11 = 0.909μ.

Method :

The slides were fixed on the stage of the microscope and the cells were focused. Cells were selected from the field of vision, beginning at one end of the smear, moving horizontally and at the other end moving downwards to the next level and again moving horizontally in the opposite direction.

Only cells that were fully included in the field of vision and with clearly defined cellular and nuclear outlines were selected, Cells that were clumped or folded were not considered for the analysis.

Measurement of the nuclear and cytoplasmic diameter was obtained in two perpendicular axis of the nuclei and cells. The average of the values from both axis was calculated and recorded as the mean nuclear diameter (ND) and the mean cytoplasmic diameter (CD). The ratio of the nuclear diameter to cytoplasmic diameter was calculated and record as the N:C ratio . 25 cells were selected from each slide and their nuclear and cytoplasmic diameters were determined.

STATISTICAL ANALYSIS:

Analysis of variance [ANOVA] was performed for the four groups [S,C,S+C and NU]. Comparison of the CD and ND, between groups was made using students independent t-test.

In this study ,$P \leq 0.05$ was considered as significant.

RESULTS

Table - 1

Number of Samples in Each Group

No	Group	Males	Females	Total
1.	Control (Normal buccal Mucosa)	10	10	20
2	Smokers	20	-	20
3	Chewers	10	10	20
4	Smokers + Chewers	20	-	20

Table - 2

Age Distribution of Samples

No	Age	Sex		Total
		Male	Female	
1	Below 30	9	4	13
2	31-40	12	6	18
3	41-50	17	8	25
4	Above 50	22	2	24

Table – 3

Comparison of cell diameter between tobacco users and non-users.

No	Group	Cell Diameter (CD)	
		Mean (μ)	**Standard Deviation**
1	Normal [a]	51.74	1.36
2	Smokers[b]	50.80	0.70
3	Chewers[b]	50.81	1.12
4	Smokers + Chewers [b]	50.47	0.89

F Value = 5.482

P Value = 0.002

Inference: The variation between (a) and (b) groups is significant at 1% level.

Table – 4

Comparison of cell Nuclear diameter between tobacco users and non-users.

No	Group	Cell Diameter (CD)	
		Mean (μ)	**Standard Deviation**
1	Normal [(a)]	8.42	0.21
2	Smokers[(b)]	8.80	0.25
3	Chewers[(b)]	8.85	0.26
4	Smokers + Chewers [(b)]	8.88	0.32

F Value = 12.77

P Value = 0.001

Inference: The variation between (a) and (b) groups is significant at 5% level.

Table – 5

Mean Cell Diameter of Age groups of tobacco users

Age Group	Male		Female		F -Value	P- Value
	Mean	SD	Mean	SD		
Below 30	50.52	7.00	50.11	6.35	0.65	0.52
31-40	49.18	5.41	49.46	6.29	0.81	0.42
41-50	50.24	2.35	50.08	5.82	2.43	0.06
Above 50	49.72	4.17	49.57	5.27	0.92	0.37
	F = 2.44 **P = 0.07**		**F = 1.98** **P = 0.12**			

Table – 6

Mean Nuclear Diameter of Age groups of tobacco users

Age Group	Male		Female		F -Value	P- Value
	Mean	SD	Mean	SD		
Below 30	7.81	0.93	7.88	1.04	0.24	0.81
31-40	7.99	0.87	8.28	0.72	1.14	0.26
41-50	8.28	0.59	7.85	0.48	2.50	0.06
Above 50	8.00	0.9	8.11	0.85	0.85	0.63
	F = 1.29 **P = 0.28**		**F= 1.28** **P= 0.29**			

Table – 7

Comparison of Nuclear diameter between male and female control (non user of tobacco)

Sex	Nuclear diameter		F-Value	P- Value
	Mean	SD		
Male	8.51	0.17	1.96	0.065
Female	8.33	0.22		

Table – 8

Chewer group

Sex	Nuclear diameter		F-Value	P- Value
	Mean	SD		
Male	8.83	0.16	0.25	0.806
Female	8.86	0.34		

Table – 9

Comparison of Cell diameter between males and females (Control Group)

Sex	Nuclear diameter		F-Value	P- Value
	Mean	SD		
Male	51.43	1.22	1.00	0.329
Female	52.04	1.47		

Table – 10

Chewer Group

Sex	Nuclear diameter		F-Value	P- Value
	Mean	SD		
Male	50.58	0.85	0.89	0.384
Female	51.03	1.34		

Discussion

Exfoliative cytology is the study of cells which are shed off as a result of normal maturation processes as well as scraped off from the surface. It is a painless non-invasive, less time consuming procedure and causes little discomfort to the patient. The exfoliated cells can be analyzed qualitatively as well as quantitatively. The qualitative methods include the assessment of cellular and nuclear atypias, staining patterns, nuclear aggregation, chromatin clumping etc. Since all these were subjective in nature, the use of oral exfoliative cytology in clinical practice declined. The more recent application of quantitative techniques have redefined the clinical role of cytology.

Quantitative exfoliative cytology include the analysis of cellular and nuclear morphology, cellular and nuclear areas, nuclear – cytoplasmic ratio, quantification of nuclear DNA content, optical density etc. Numerous studies were conducted in various conditions and disease processes.

The present study was undertaken to assess the morphometric changes in cell diameter (CD), and nuclear diameter

(ND) of squames from clinically normal buccal mucosa and compare with it non-users of tobacco and users of tobacco who smoke, chew and practice both habits. The present study was also undertaken to findout if any correlation exists between the cell diameter and nuclear diameter regarding gender and age and the tobacco habits.

Our study consists of total of 80 subjects. The control group (non – users of tobacco), smokers, chewers, smokers + chewers each contain 20 subjects. Out of the 80 subjects, 20 were females. That includes 10 females in the control group and 10 in the chewers group. There are no females in the tobacco smoker group and combined habits group. This may be because tobacco smoking habit is very rare among Indian females.

Regarding the age distribution of the samples 9 males and 4 females were below 30 years old. 12 males and 6 females were in between 31 and 40.17 males and 8 females were between 41 and 50. 22 males and 2 females were above 50 years of age. Thus a total of 13 subjects were below 30 years, 18 between 31 and 40, 25 between 41 and 50 and 24 were above 50 years old.

In our study the individual smears from normal subjects showed a wide variation in the size of the cells and nuclei. This was explained on the basis of cell development and maturation by

lee at al (1973). Variation in the cell and nuclear size was also reported by **Boddington et al (1953)** and **Goldsby et al (1964).**

The mean cell diameter and nuclear diameter in our study did not differ significantly with aging or between sexes. This finding is consistent with the study of **Cowpe et al** (1985) and **Wolff et al** (1991). They suggested that aging does not produce changes in the cell and nuclear dimension.

In our, study the mean cellular diameter of normal, smokers, chewers and smoker + chewer group are 51.74 $\pm$ 1.36, 50.80 $\pm$ 0.70, 50.81 $\pm$ 1.12 and 50.47 $\pm$ 0.89 respectively. The mean nuclear diameter for the corresponding groups are 8.42 $\pm$ 0.21, 8.80 $\pm$ 0.25, 8.85 $\pm$ 0.26 and 8.88 $\pm$ 0.32 respectively. There is statistically significant difference between the control group and the tobacco users (P=0.002) regarding the cell diameter and (P=0.001) regarding the nuclear diameter. Both are significant at 1% level. Thus there is a decrease in the cellular size and increase in the nuclear size in the tobacco user groups.

Increase in the nuclear size for smokers have been reported by **Ogden et al** (1990). **Hillman et al** (1976) reported that nuclear size increased in smokers and an opposite trend was found in individuals who smoked more than two packets daily.

Ramaesh et al (1999) reported increase in nuclear size in smokers, chewers and combined habit groups compared to controls. Our reports are consistent with the findings of **Ogden et al** and **Ramaesh et al**.

Ogden et al (1990) reported no significant difference in cell size of smokers compared to non-smokers. As opposed to this, **Hillman et al** (1976) reported a greater cell size in smokers than non-smokers. Although both the studies reported an increase in nuclear size in tobacco smoking, the change in cell size was reported only be **Hillman et al** (1976). **Ramaesh et al** (1999) reported decrease in the cell size in the tobacco user groups (Smokers, chewers and combined habit). Our report is consistent with the report of **Ramaesh et al** and differs from the report of **Ogden et al**.

Increase in nuclear size and reduction in cell size have been reported to be early malignant changes seen in oral epithelial cells as per the reports of **Cowpe et al** (1988) and **Ramaesh et al** (1988). Similar changes (increase in ND and reduction in CD) were observed in the buccal cells exposed to the habit of tobacco chewing suggest that this habit may be related to the development of oral premalignant and malignant lesions.

Though the oral mucosa appears clinically normal in tobacco users, the cytomorphometrical changes noted in the smears resemble those seen in association with premalignant and malignant lesions of the oral mucosa. The inference from this study supports the cause - effect relationship between the tobacco use and oral cancer as implied in epidemiological studies.

SUMMARY AND CONCLUSION

This study was done to assess the cell diameter (CD) and nuclear diameter (ND) of squames in clinically normal buccal mucosa of patients having tobacco habits and to correlate with gender and age.

The cell and nuclear size of normal subjects showed wide variation. ND ranged from 7.8 μ to 8.5μ and CD ranged from 46.20μ to 72.61μ.

The mean cell diameter in the tobacco non-user group (51.ur ± 1.36) is significantly higher than the various tobacco user groups. The mean cell diameter of tobacco smokers, chewers and combined habit groups are 50.80 ± 0.70, 50.81 ± 1.12 and 50.47 ± 0.89 respectively. Though the mean cell diameter slightly decreased from tobacco smoker to chewer to combined habit group.

The mean nuclear diameter in the tobacco non-user group (8.42 ± 0.21) is significantly lower than the various tobacco user groups. The mean nuclear diameter of tobacco smokers, chewers and combined habit groups are 8.80 ± 0.25 , 8.85 ± 0.26 and 8.88 ± 0.32 respectively. The mean nuclear diameter slightly increased from the tobacco smoker to chewer to combined habit group.

The reduction in the cell diameter and an increase in the nuclear diameter in the tobacco user groups show that these parameters could be early indications towards dysplasitc changes.

Comparison of cell diameter (CD) and nuclear diameter (ND) between males and females in different study groups showed no significant difference by students independent t – test.

Comparison of cell diameter (CD) and nuclear diameter (ND) between no habit group and tobacco user groups showed significant difference. No significant difference was seen in the cell diameter (CD) and nuclear diameter (CD) between the different tobacco user groups in the study by one-way ANOVA and multiple Range test by Tukey HSD procedure.

Comparison of cell diameter (CD) and nuclear diameter (ND) between different age groups (<30,31-40, 41-50, >51 years) by one-way ANOVA and multiple range test by Tukey HSD procedure showed no significant difference.

This prelimnary study shows that various tobacco habits have significant alteration in the mean cell diameter and mean nuclear diameter of squames of buccal mucosa.

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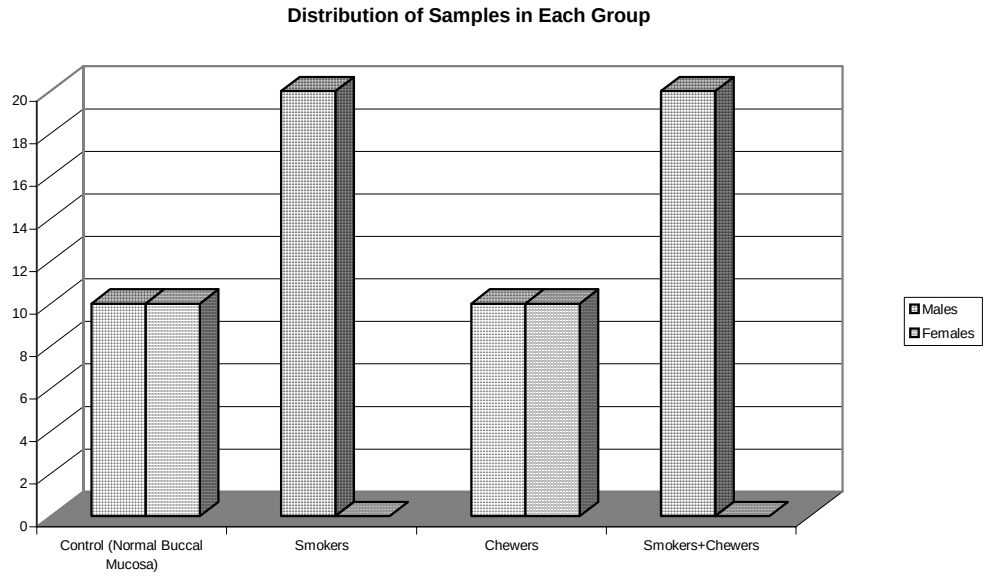
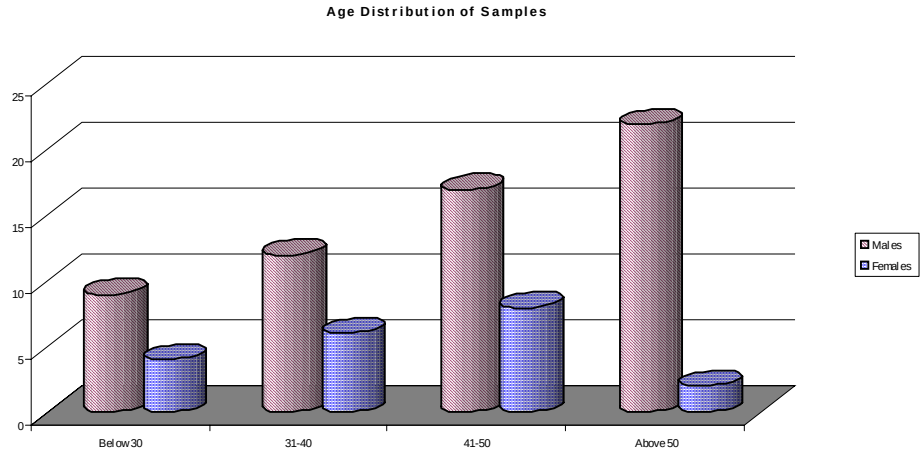

Distribution of Samples in Each Group
20
18
16
14
12
10
8
6
4
2
0
Control (Normal Buccal Mucosa)
Smokers
Chewers
Smokers+Chewers
Males
Females


Age Distribution of Samples
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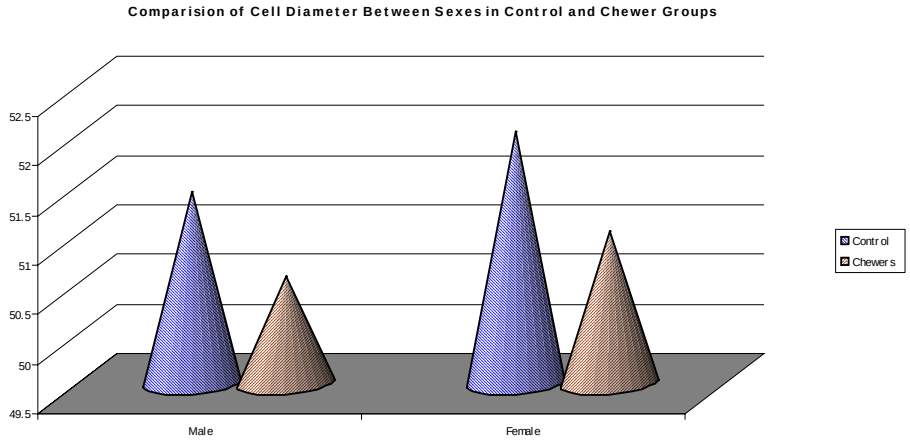
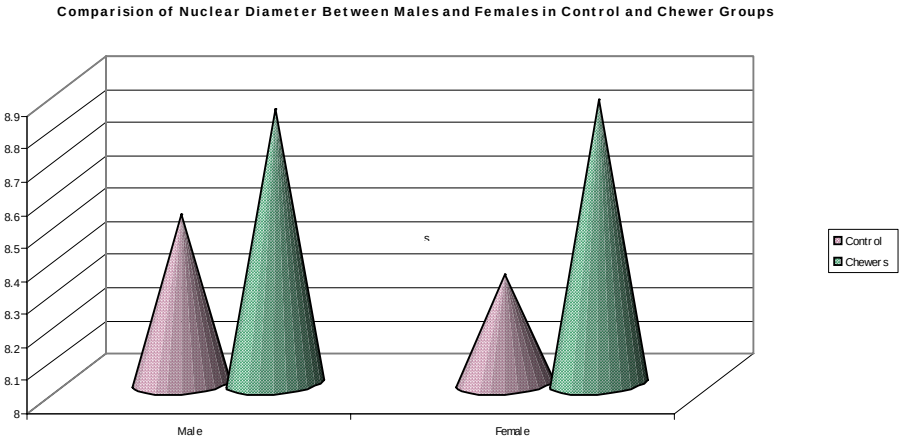
20
15
10
5
0
Below 30
31-40
41-50
Above 50
Males
Females


Comparision of Cell Diameter Between Sexes in Control and Chewer Groups
52.5
52
51.5
51
50.5
50
49.5
Male
Female
Control
Chewers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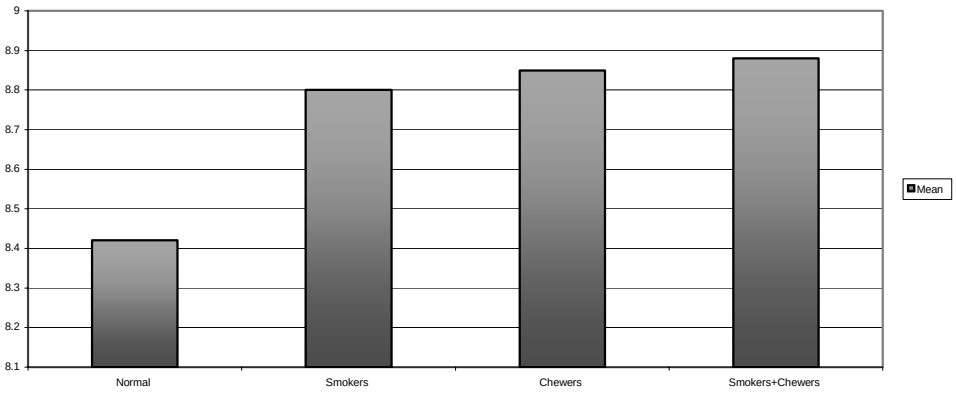
8.9
8.8
8.7
8.6
8.5
8.4
8.3
8.2
8.1
8
Male
Female
Control
Chewers

Bar Diagram Depicting the Mean Cell Diameter of Different Study Groups



Bar Diagrm Depicting the Mean Nuclear Diameter of Different Study Groups